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Role of the Sigma Factor in Transcription Initiation in the Absence of Core RNA Polymerase

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SUMMARY

Sigma factors (σs) are bacterial transcription factors that bind core RNA polymerase (RNAP) and direct transcription initiation at cognate promoter sites. However, most of their functions have been investigated in the context of RNAP. This has made the exact function of σ, and the importance of core RNAP in modulating σ function, ambiguous. Here we identi *Bacillus subtilis* mutant σ^A that is independe ly capable of specific binding and melting of t promoter DNA. Interestingly, spe ic a d inde pendent promoter binding of σ s suffi ent for the temperature- and Mg^{2+}-i dependent melting of promoter DNA aro nd th transcription start site, in contrast t the temperature- and Mg^{2+}-dependent me ing by RNAP a und the promoter −10 element. Thus core RNAP is able to negati ely modulate the σ-initiated melting of th transcription start site and, by sensing th chang s in temperature and Mg^{2+} concentrati to regulate the efficiency of promoter me ing.

INTRODUCTION

Transcription initiation is the first step in gene expression and a major point for gene regulation. σ of RNA polymerase holoenzyme (RPase; $\sigma\alpha_2\beta\beta'$) confers the specificity of promoter recognition and initiates transcription (Burgess et al., 1969; Gardella et al., 1989; Siegele et al., 1989; Vassylyev et al., 2002; Zuber et al., 1989). It is also involved in open complex formation (Juang and Helmann, 1994; Panaghie et al., 2000; Rong and Helmann, 1994; Tomsic et al., 2001), abortive transcription (Hernandez et al., 1996; Sen et al., 1998), promoter-proximal pausing (Ring et al., 1996), and the regulation of gene expression (Hernandez and Cashel, 1995; Landini and Busby, 1999; Rhodius and Busby, 2000).

The σ can be divided into four conserved regions, with each containing two to four subregions. The conserved regions 2.4 and 4.2 have been thought to recognize, respectively, the −10 and −35 consensus elements of promoter (Dombroski et al., 1990; Gardella et al., 1989; Kahn and Ditta, 1991; Keener and Nomura, 1993; Marr and Roberts, 1997; Siegele et al., 1989). However, multiple switches appear to be built into σ to ensure that it doesn't bind the promoter DNA by itself (Borukhov and Severinov, 2002). The inhibition can be assumed to be attributed to the direct interaction between regions 1.1 and 4.2 of the σ (Dombroski et al., 1992, 1993). However, NMR spectroscopy and segmental isotopic labeling studies of a *Thermotoga maritima* σ have revealed that the inhibition is owing to an indirect steric and/or electrostatic mechanism, which makes the distance between regions 2.4 and 4.2 of the σ inappropriate for simultaneous interaction with their respective promoter elements (Camarero et al., 2002). It is the interaction between the σ and the core RNA polymerase (core RPase; $\alpha_2\beta\beta'$) that alleviates the distance constraint and allows efficient promoter binding. The interaction includes at least the contact between the flap domain of β of the core RPase and region 4 of the σ (Kuznedelov et al., 2002; Murakami et al., 2002; Vassylyev et al., 2002; Zhang et al., 1999) as well as between the coiled-coil domain of β′ of the core RPase and region 2.2 of the σ (Young et al., 2001). Once the promoter binding has taken place, RPase melts the duplex promoter DNA from roughly −11 to +2, relative to the transcription start site, +1 (Kirkegaard et al., 1983; Siebenlist, 1979). Regions 2.3 and 2.4 of the σ may promote the melting by capturing the nontemplate bases from the −10 promoter element, with which the bases are flipped out of the double helix due to thermal motion (Juang and Helmann, 1994; Marr and Roberts, 1997; Panaghie et al., 2000; Tomsic et al., 2001; Young et al., 2004).

Up to now, most studies of σ function were performed in the context of RPase. This has made it difficult to identify the exact function of the σ and the importance of core RPase in the functioning of the σ. Our discovery of a *Bacillus subtilis* mutant σ^A capable of specific binding and melting of the promoter DNA independent of core RPase, as presented in the paper, has allowed us to clarify the ambiguities.

Here, we report how such a novel mutant σ^A was discovered and how it was used to clarify the ambiguities.

RESULTS

Specific and Nonspecific Binding of the N-Terminally Truncated σ^A to Promoter DNA

In our previous study of the promoter DNA binding capabilities of the wild-type (WT) and truncated σ^A-RPases of *B. subtilis*, we accidentally observed that σ^A, with region 1.1 and a few amino acid residues removed at the N-terminal part of region 1.2 (Figure 1A), had a potential to bind the G3b promoter DNA of *B. subtilis* ϕ29 phage (Hsu et al., 2004). The binding was strong enough to be monitored by electrophoretic mobility shift assay (EMSA). To confirm this observation, seven truncated σ^A, lacking in 100 to 109 amino acids at their N terminals, were adopted to repeat the experiment. As shown in Figure 1B, two extra protein-DNA complexes, in addition to that of the RPase-promoter DNA complex, were observed when core RPase was saturated with a 10 time molar excess of the truncated σ^A in the presence of 0.1 μM heparin, which helped to eliminate the formation of σ^A-DNA aggregate (Hsu et al., 2004). These two complexes were also detectable when core RPase was omitted, thus indicating that both of them are formed by binding of the truncated σ^A to the G3b promoter DNA. Thus, in contrast to the WT σ^A, the seven truncated σ^A were found to be capable of independent binding to the promoter DNA.

To characterize the two distinct σ^A-DNA complexes in greater detail, we chose the SND100-σ^A, which is deficient of the entire 1.1 region of the σ^A and is as active as the WT both in vivo and in vitro (Hsu et al., 2004), for further DNA binding analysis. In this analysis, the molar ratio of SND100-σ^A to G3b promoter DNA was varied from 100:1 to 3200:1. Under these conditions, at least three σ^A-promoter-DNA complexes were observed (Figure 1C). The fastest migrating complex, complex I, which was detectable under all conditions, was minor and smearing. This suggested that it may dissociate either transiently or is unstable. Complex II became more abundant as the molar ratio of σ^A to DNA increased from 100:1 to 800:1. Thereafter, it was gradually transformed into complex III (or further into complex IV?). The stepwise formation of complexes II and III indicated that they are formed by consecutive binding of SND100-σ^A to the promoter DNA.

To verify the above interpretation, the decays of both complexes II and III were analyzed. To this end, the preformed complex III was challenged with an increasing concentration of nonpromoter DNA. As shown in Figure 1D, complex III was converted into complex II when the molar ratio of nonpromoter DNA to promoter DNA was 9:1. However, further conversion of complex II into free promoter DNA required a much higher concentration of the nonpromoter DNA. The stepwise formation and decay of complex III suggested that it is formed by sequential specific (promoter) and nonspecific (nonpromoter) binding of σ^A to G3b promoter DNA; and that complex II is the specific promoter binding complex. To strengthen the idea that complex II is a specific σ-promoter complex, we analyzed the fractional retention of complex II as a function of increasing the molar ratio of the competitor (cold G3b promoter DNA or nonpromoter DNA) to the labeled G3b promoter DNA. As shown in Figure 1E, we obtained the lower fractional retention of complex II using the challenge of the specific (G3b promoter DNA) rather than the nonspecific competitor (nonpromoter DNA), which further supports the argument that complex II is a specific σ-promoter DNA complex.

The Footprints of σ^A and σ^A-RPase on G3b Promoter DNA

A DNase I footprinting assay provided the conclusive evidence that SND100-σ^A specifically binds to G3b promoter DNA. To carry out this assay, we screened for the condition required for the formation of specific complex II. As shown in Figure 2A, complex II was formed specifically when the molar ratio of SND100-σ^A to G3b promoter DNA was 800:1 and the concentration of heparin was about 0.6 to 1.0 μM. Under the specific binding condition, we observed clear footprints encompassing −35 and −10 elements (from +1 to −45 on the nontemplate strand and +1 to −5 on the template strand DNA) of the G3b promoter DNA for SND100-σ^A (Figure 2B), which indicated that SND100-σ^A carries out specific promoter DNA binding activity. However, we also observed a change in the digestion kinetics of the G3b promoter DNA downstream of the transcription start (+1) site on the template strand DNA. We have nevertheless inferred that this change is a result of conformational change of G3b promoter DNA in response to specific SND100-σ^A binding, not to extra nonspecific SND100-σ^A binding, since complex II forms specifically under the experimental conditions explained above (Figure 2A) and since the change in the digestion kinetics of DNA downstream of the transcription start site persists unless the concentration of heparin is higher enough (>1.2 μM) to remove the specifically bound SND100-σ^A from the complex II and convert it into free promoter DNA (Figure 2C). We also carried out the same footprinting assay without the use of heparin. In the absence of heparin, we noted extended footprints from −50 to +40 on the nontemplate strand and −90 to +30 on the template strand DNA for SND100-σ^A. These were much bigger than those observed for both the WT-σ^A- and SND100-σ^A-RPases (Figure 2B). Thus, in addition to specific binding to G3b promoter DNA, SND100-σ^A is also able to bind to G3b promoter DNA nonspecifically.

−10 and −35 Promoter Elements Are Critical for Specific and Efficient Binding of σ^A to G3b Promoter DNA

To determine which promoter element(s) is critical for specific and efficient SND100-σ^A binding, we constructed four G3b promoter mutants with base substitutions at either the −10, TG, or −35 elements (Figure 3A). The resulting mutant promoters were then used to investigate

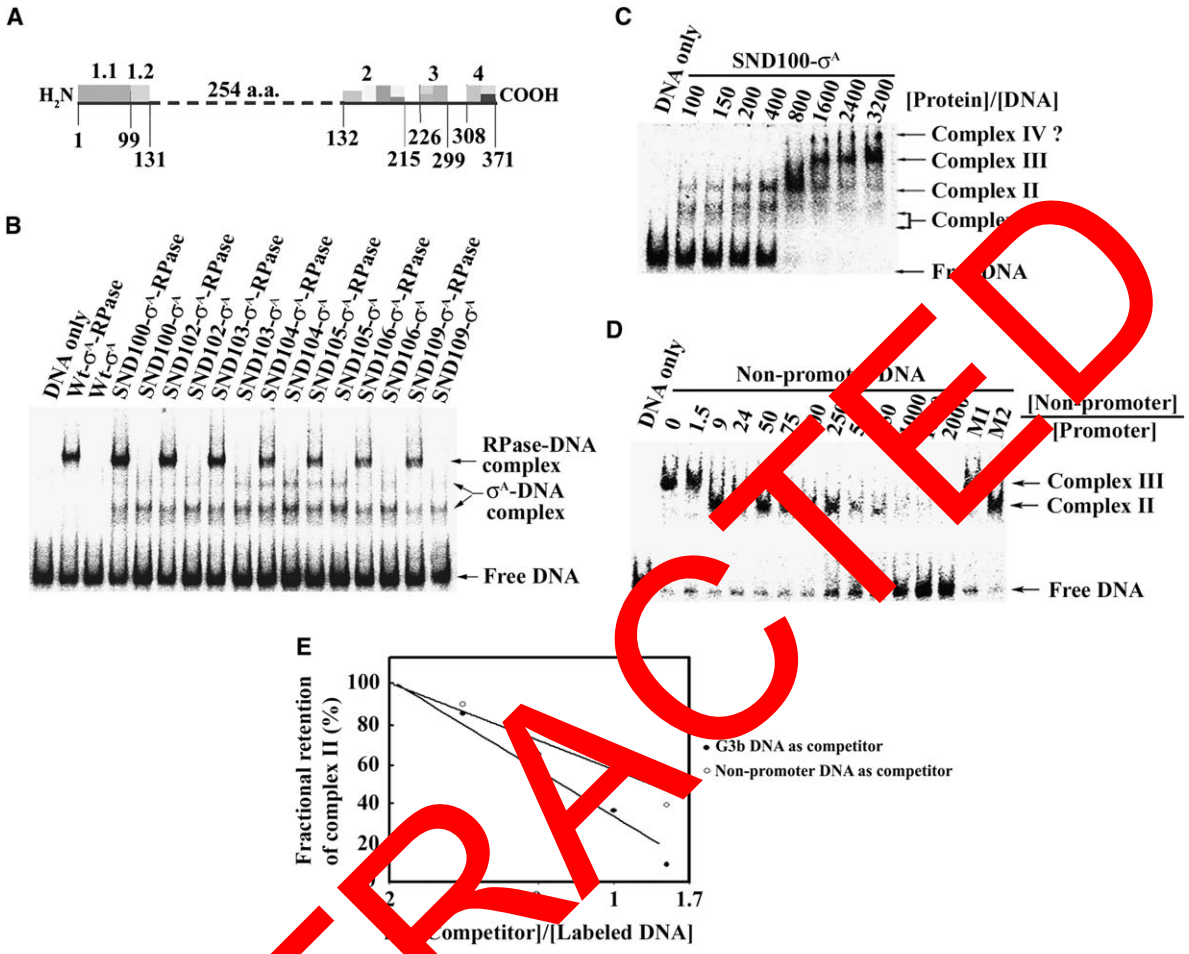

Figure 1. Formation and Decay of the Truncated σ^A-Promoter DNA Complexes

(A) The conserved regions of *B. subtilis* σ^A. Region 1 of σ^A is divided into subregions 1.1 and 1.2. The 254 amino acid spacer as indicated by the broken line is absent from *B. subtilis* σ^A.

(B) The promoter DNA binding ability of the N-terminally truncated σ^A. SNDX-σ^A indicates mutant σ^A with X (100, 102, 103, 104, 105, 106, or 109) amino acid residues at the mutant N terminus of the WT-σ^A deleted. Each binding mixture contained 1 nM 5′ end-labeled ϕ29 phage G3b promoter DNA and either 250 nM SNDX-σ^A or 25 nM SNDX-σ^A-RPase reconstituted from 250 nM SNDX-σ^A and 25 nM core RPase. The binding reaction was carried out in the presence of 0.1 μM heparin to avoid aggregation of the protein-DNA complex. The G3b promoter DNA was 181 bp in length and encompassed the sequences from −112 to +69 of the promoter.

(C) Formation of the SND100-σ^A and G3b promoter-DNA complexes. Each binding mixture comprised 1 nM 5′ end-labeled G3b promoter DNA and an indicated concentration (nM) of SND100-σ^A supplemented with 0.1 μM heparin.

Decay of SND100-σ^A and G3b promoter-DNA complexes. To generate complex III, 1 nM G3b promoter DNA was mixed with 1600 nM SND100-σ^A supplemented with 0.1 μM heparin. To analyze the stability of complexes III and II, the preformed complex III was challenged with an indicated concentration (nM) of nonpromoter DNA, which was the *tgbp2* cDNA from *Bamboo mosaic virus*. M1 and M2 represent complex III and complex II, respectively.

(E) Fraction retention of complex II as a function of increasing molar ratio of competitor to labeled promoter DNA. The preformed complex II was challenged with a specific (cold G3b promoter DNA) or a nonspecific (cold *tgbp2* cDNA) competitor. The molar ratios of competitor to labeled G3b promoter DNA were 0:1, 0.1:1, 1:1, 10:1, and 50:1.

the importance of each promoter element for the binding of SND100-σ^A or SND100-σ^A-RPase to the G3b promoter through an EMSA. As shown in the top panel of Figure 3B, the formation of the RPase-promoter-DNA complex was significantly prevented by mutation at the −10, TG, or −35 elements. However, we noted that while there was significant prevention of complex II formation when there were base substitutions at either the −10 or −35 element, significant prevention of complex II formation did not occur where there were base substitutions only at the TG motif (Figure 3B, bottom panel). These results clearly demonstrated that both the −10 and −35 elements are critical for specific and efficient binding of SND100-σ^A or SND100-σ^A-RPase to G3b promoter. Moreover, the sharing of both the −10 and −35 elements for specific and efficient binding strongly suggested that a similar

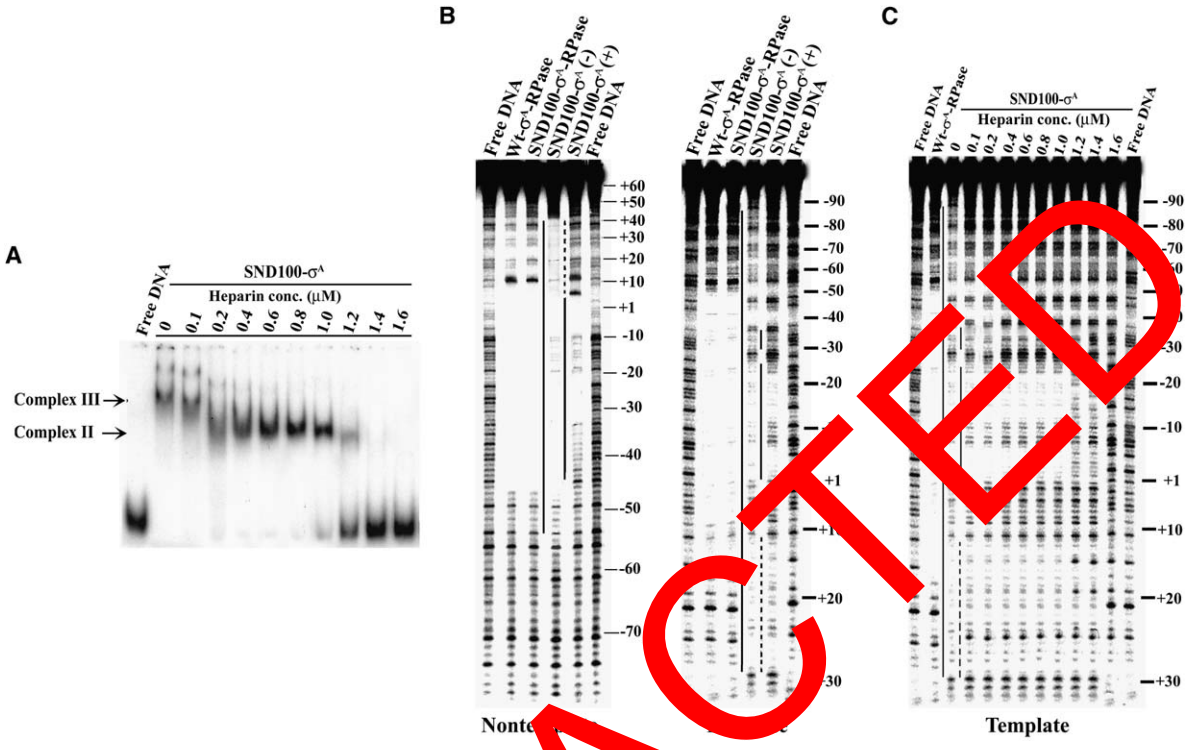

Figure 2. The Footprints of σ^A and σ^A-RPases on G3b Promo[illegible] DNA

(A) Screening of condition for specific binding of SND[illegible] to G3b p[illegible]oter DNA. The molar ratio of SND100-σ^A to G3b promoter DNA in each binding mixture was 800:1.

(B) Footprints of σ^A and σ^A-RPases on the nonte[illegible]plate and t[illegible]plate stra[illegible]DNA, respectively. The RPase was reconstituted from core RPase and a 10 time molar excess of σ^A. For footprinting a[illegible]ys, the m[illegible] of G3b promoter DNA to σ^A-RPase or to SND100-σ^A in the binding mixture was 1:100 or 1:800, respectively. The σ^A-prom[illegible]r-DN[illegible]m[illegible]x formed in the presence (+) or absence (−) of 0.6 μM heparin was digested with DNase I.

(C) Footprint of SND100-σ^A on the tem[illegible]e strand D[illegible] in the presence of various concentrations of heparin. In both (A) and (C), the numbers shown under SND100-σ^A are concentration[illegible]M) of heparin ad[illegible] into the binding mixture. The regions bound by SND100-σ^A are shown in solid lines. The broken line indicates the region ([illegible]m +[illegible] +30) that appe[illegible] to change in DNA conformation.

mechanism is adopt[illegible] by σ^A and σ^A-RP[illegible] to specifically bind to promoter DNA.

Core RPase Is Re[illegible]d for Mo[illegible]Efficient Promoter Binding [illegible]RPase

Is the [illegible]rength[illegible] specific[illegible]A[illegible]romoter binding comparable to that of [illegible]pecific σ^A-RPase-promoter binding? We clari[illegible]d thi[illegible]point by [illegible]asuring the apparent dissociation consta[illegible] (K_D) of the complex formed by G3b promoter DNA and [illegible]her SND100-σ^A or SND100-σ^A-RPase (Table S1 available [illegible]ith this article online). The dissociation constant of SND100-σ^A ($K_D \sim 8 \times 10^{-7}$ M^{-1}), being one order of magnitude higher than that of SND100-σ^A-RPase ($K_D \sim 7 \times 10^{-8}$ M^{-1}), indicated that core RPase is required for more efficient promoter binding of σ^A-RPase to take place.

σ^A Melts Promoter DNA Independent of Core RPase

One possible explanation for the change in digestion kinetics from about +10 to +30 on the template strand of the G3b promoter DNA, in response to SND100-σ^A binding (Figure 2B), was the melting of the duplex promoter DNA. Given that the open complex is relatively more resistant to challenge with single-stranded DNA analog such as heparin, we examined the resistance of complex II to heparin. In the examination, the preformed complex III was treated with various concentrations of heparin (from 0 to 1.2 μM) prior to carrying out an EMSA (Figure 4A). We removed the nonspecifically bound σ^A from the complex III with lower concentrations of heparin (no higher than 0.4 μM). However, a relatively higher concentration (at least 0.6 μM) of heparin was required for further removal of the specifically bound σ^A from complex II, which indicated that complex II is a relatively more stable complex than complex III.

To verify that complex II is an open complex, we performed $KMnO_4$-primer extension analysis, in which thymine residue on the melted DNA is most significantly modified. Remarkably, significant base modifications around −3 to +2 on the template and −7 to −6 on the nontemplate strand DNA were observed for complex II. This indicated that SND100-σ^A, by itself, melts the duplex promoter DNA (Figure 4B). However, we observed base modifications around −9 to −6 on the template and −12 to −7

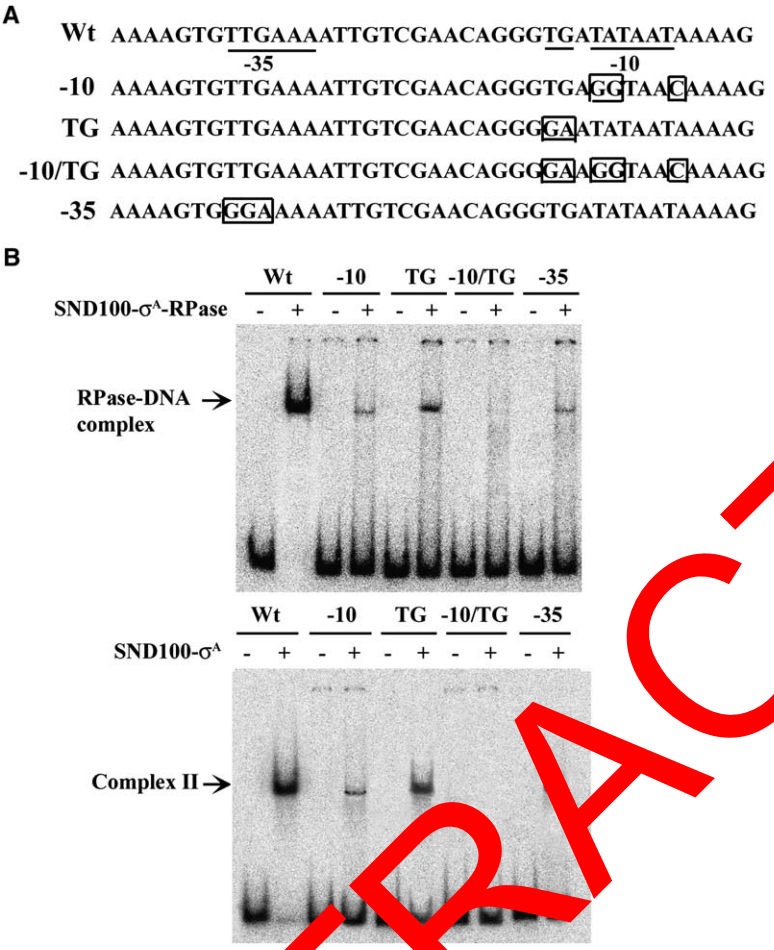

Figure 3. Effect of Promoter Mutation on Binding of σ^A or σ^A-RPase to G3b Promoter DNA

(A) Mutations of G3b promoter. The sequences of conserved −10, TG, and −35 elements of WT G3b promoter are underlined. −10, TG, −10/TG, and −35 indicate the four promoter mutants. Base substitutions in each of the mutant promoters are boxed.

(B) Effect of promoter mutation on binding of SND100-σ^A-RPase (top panel) or SND100-σ^A (bottom panel) to G3b promoter, as examined by EMSA. The molar ratio of SND100-σ^A-RPase or SND100-σ^A to promoter DNA is 40:1 or 800:1, respectively. The heparin concentration was 50 μM in the binding mixture containing SND100-σ^A and promoter DNA.

on the nontemplate strand DNA for the G3b promoter DNA in response to the binding of SND100-σ^A-RPase or WT-σ^A-RPase (Figure 4B). Thus, SND100-σ^A is able to melt G3b promoter DNA independent of core RPase around the transcription start site, a little way downstream from the G3b promoter DNA melted by σ^A-RPases.

The Requirement of at Least Three Identical Aromatic Amino Acid Residues for Promoter Melting by σ^A and σ^A-RPase

To clarify that the promoter melting by σ^A is mechanistically related to that mediated by σ^A in the context of RPase and that it is biologically relevant, seven alanine substitutions for aromatic amino acid residues (F178A, Y180A, Y184A, F186A, Y189A, W192A, and W193A), which appear to decrease the ability of σ^A-RPase to melt promoters (Juang and Helmann, 1994, 1995), were introduced, respectively, into region 2.3 of the SND100-σ^A. The resultant mutant σ^A was then used to analyze the effects of alanine substitutions on the binding and melting of G3b promoter DNA by SND100-σ^A. As shown in Figure 5A, the effects of alanine substitutions on promoter binding can be grouped into three classes. The first class (F178A and F186A) signifies the elimination of most of the promoter binding activity of SND100-σ^A. The second (184A and W192A) signifies the partial reduction of the promoter binding activity, while the third (Y180A, Y189A, and W193A) signifies that the promoter binding activity did not alter significantly. However, all of the three classes of mutations significantly diminished the $KMnO_4$ reactivity, by around −3 to +2, on the template strand DNA of the G3b promoter (Figure 5C) despite their different effects on promoter binding. The prevention of promoter melting by the first two classes of substitutions (F178A and F186A; 184A and W192A) seems to have been attributable at least partly to the decreased promoter binding activity of the mutant σ^A. Nevertheless, the prevention of promoter melting by the third class (Y180A, Y189A, and W193A), which did not significantly alter the promoter binding activity of the σ^A, revealed that Y180, Y189, and W193 at least participate in the melting of the G3b promoter by SN100-σ^A.

The effects of alanine substitutions for aromatic amino acid residues on promoter binding and melting by SND100-σ^A-RPase were also examined. F178A and F186A clearly reduced both the binding of SND100-σ^A-RPase to the G3b promoter (Figure 5B) and the $KMnO_4$

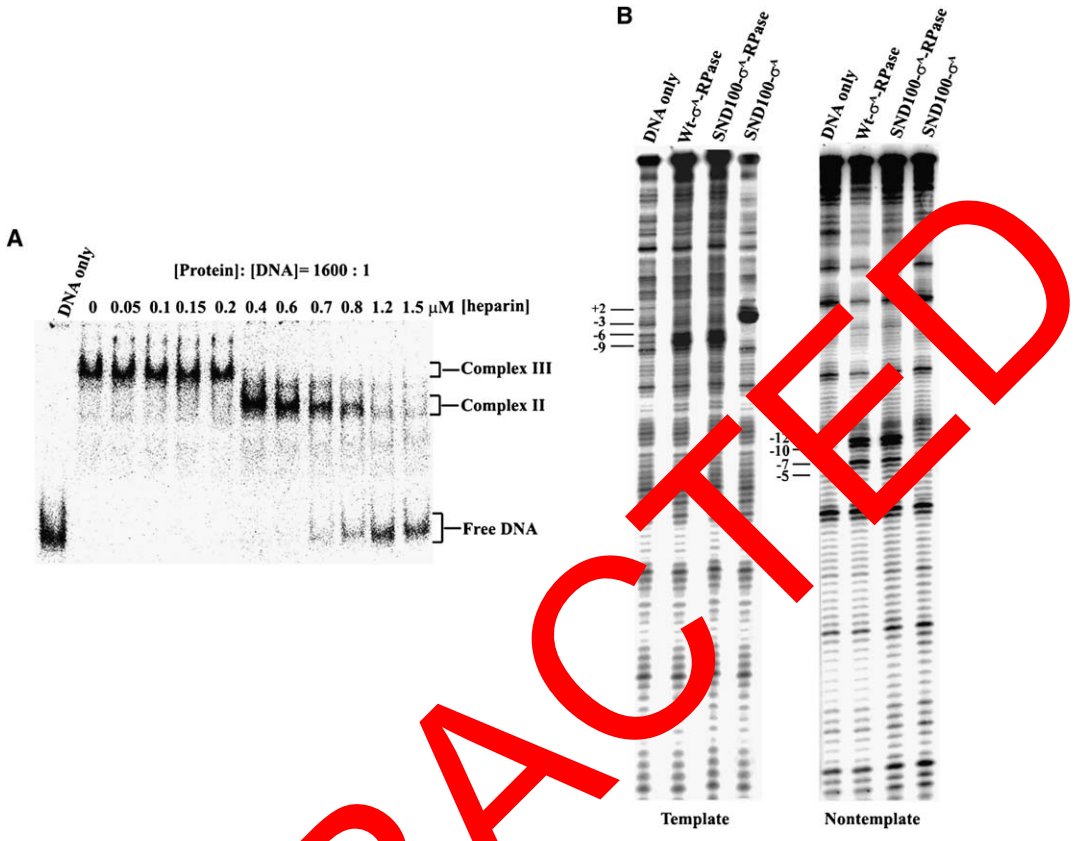

Figure 4. Melting of the G3b Promoter D[illegible] by σ^A a[illegible]σ^A-RPases

(A) Heparin resistance of the σ^A-promoter-DNA c[illegible]plexe[illegible] The pre[illegible] complex III prepared by mixing 1600 nM SND100-σ^A with 1 nM G3b promoter DNA was treated with various co[illegible]ntrations [illegible]eparin (from 0 to 1.5 μM, as indicated) before being run on a native polyacrylamide gel.

(B) $KMnO_4$-primer extension analyses[illegible]he molar ratio [illegible]^A-RPases or SND100-σ^A to G3b promoter DNA in the binding mixture was 100:1 or 800:1, respectively. Both template and n[illegible]te strand DNA[illegible] the σ^A-DNA complexes formed in the presence of 0.6 μM heparin were probed with $KMnO_4$. The numbers shown o[illegible]he left a[illegible]ositions relative to the transcription start site of G3b promoter.

reactivity at −9[illegible]o −6 o[illegible] the template strand DNA (Figure 5C). U[illegible]r the [illegible]me experimental conditions, Y180A, Y184A, Y[illegible]A, [illegible]W192A, a[illegible]W193A did not have any sig[illegible] effect[illegible] σ^A-R[illegible]se binding (Figure 5B). How[illegible]er, they [illegible]id reduc[illegible] $KMnO_4$ reactivity at −9 to −6[illegible]n the te[illegible]plate DNA strand (Figure 5C). Taken toget[illegible] the[illegible] resu[illegible]emonstrated that at least three identic[illegible]mino acid residues, Y180, Y189, and W193, are critica[illegible] SND100-σ^A and SND100-σ^A-RPase in melting the G3b p[illegible]moter. The similarity in the requirement for amino acids in promoter melting indicated that the promoter melting by σ^A is mechanistically related to that mediated by σ^A in the context of RPase and is indeed biologically relevant.

The Two-Step Promoter Melting by σ^A-RPase

The melting of G3b promoter DNA by σ^A-RPase around the promoter −10 element (from −12 to −6), rather than melting by SND100-σ^A around the transcription start site (from −7 to +2), suggested that the promoter DNA is melted by σ^A-RPase in two separate steps and that core RPase is capable of modulating the accessibility of the transcription start site, probably by trapping the σ^A-RPase and promoter DNA complex in an initial opening stage. To make the transcription start site accessible in the σ^A-RPase and promoter DNA complex, initiation nucleoside triphosphate (NTP) was once thought to be required. However, neither guanosine triphosphate (GTP), the initiation nucleotide, nor adenosine triphosphate (ATP), uridine triphosphate (UTP), or cytosine triphosphate (CTP) was able to trigger separately the propagation of melting (see Figure S5). Only in the presence of both ATP (−1 nucleotide) and GTP (+1 nucleotide) was the propagation of melting possible (Figure 6B, bases indicated by Δ). However, some transcription might also occur under this condition since base modification further downstream of the +2 position was observable. Our finding that both −1 and +1 nucleotides were required for propagation of melting was unexpected. These unforeseen results revealed that σ^A-RPase melts G3b promoter DNA in two separate steps during transcription initiation, of which the second step is nucleotide dependent.

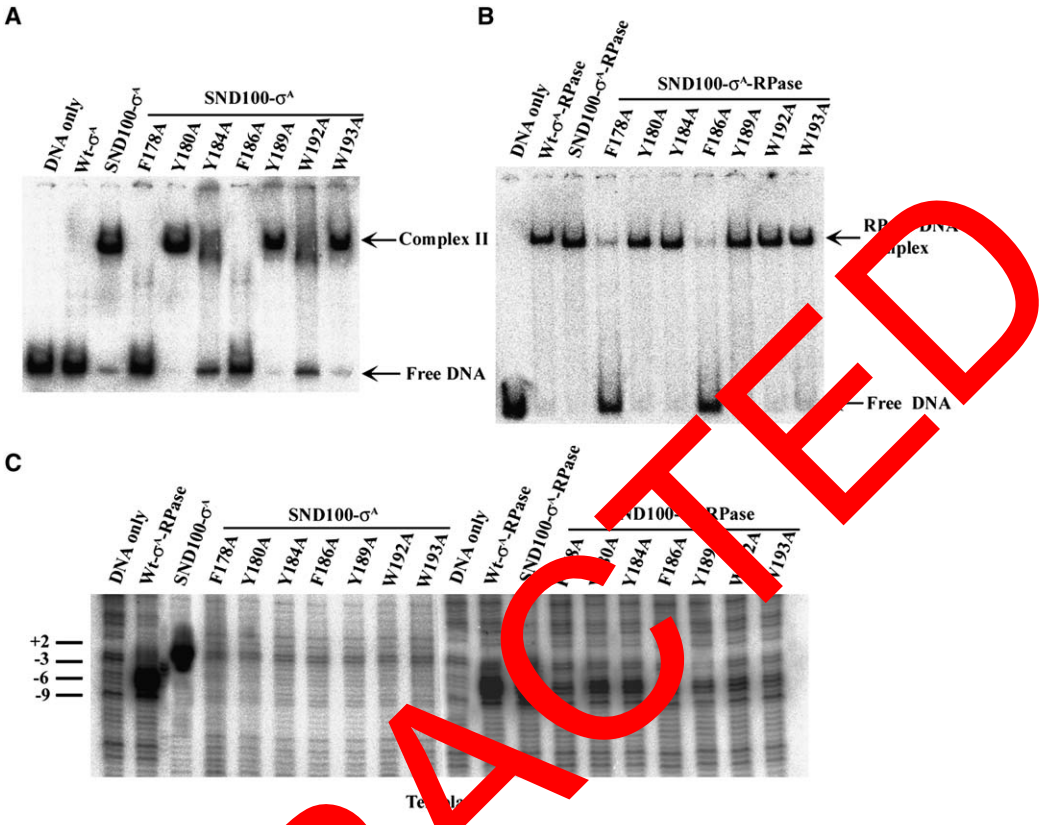

Figure 5. Promoter DNA Binding and M[illegible]ding by σ^A or σ^A-RPa[illegible] as Influenced by Aromatic Amino Acid to Alanine Substitution in Region 2.3 of σ^A

(A) Effect of alanine substitution in regio[illegible]2.3 of [illegible]σ^A on σ^A bin[illegible]ng to G3b promoter. The molar ratio of SND100-σ^A to G3b promoter DNA in the binding mixture was 800:1. The ala[illegible]e substitut[illegible]are as indicated at top of the figure.

(B) Effect of alanine substitution i[illegible]ion 2.3 of SND[illegible]-σ^A on binding of SND100-σ^A-RPase to G3b promoter. The SND100-σ^A-RPase was reconstituted from core RPase and [illegible]0 t[illegible] molar excess o[illegible]ND100-σ^A. The molar ratio of SND100-σ^A-RPase to G3b promoter DNA was 40:1 in the binding mixture. The amino acid substit[illegible]ns are as indicated at top of the figure.

(C) Effect of alanine sub[illegible]ution in region 2.[illegible] SND100-σ^A on G3b promoter melting by SND100-σ^A and SND100-σ^A-RPase. For the promoter-melting assay, the molar [illegible]o of SND100-σ^A or SN[illegible]0-σ^A-RPase to G3b promoter was 800:1 or 100:1, respectively. The concentration of heparin in the binding mixture o[illegible]ND100-σ^A [illegible]d G3b promoter DNA was 1.2 μM. The $KMnO_4$ reactivity of the template strand DNA of G3b promoter, as influenced by each mutat[illegible]is shown

Prom[illegible]elting [illegible] σ^A Is[illegible]emperature and Mg^2+ Inde[illegible]pende

[illegible]has been [illegible]eported that RPase melts the promoter DNA i[illegible] tem[illegible]erature[illegible]endent manner (Suh et al., 1993). It has [illegible] been unclear, however, whether the melting of promot[illegible]DNA by σ is also temperature dependent. We undertook [illegible]is question by analyzing the effect of temperature change on promoter melting by SND100-σ^A or σ^A-RPase. As shown in Figure 7A, we detected that temperature change had no effect on SND100-σ^A in base modification around the transcription start site on the template strand DNA. However, we observed that temperature change from 4°C, 15°C, 25°C, to 37°C had a pronounced effect on both WT-σ^A- and SND100-σ^A-RPases in base modification at the −10 element of promoter. This indicates that it is core RPase that is responsible for temperature dependency in promoter melting.

We also examined the effect of Mg^{2+} on base modification on the template strand DNA of the G3b promoter since Mg^{2+} has been found to enhance DNA melting around the transcription start site (Suh et al., 1993). Similar to the temperature effect, melting of the G3b promoter DNA around the transcription start site by SND100-σ^A was not affected by the presence of Mg^{2+}. In contrast, melting of the promoter −10 element by σ^A-RPase was Mg^{2+} dependent (Figure 7B). The difference in Mg^{2+} dependence between SND100-σ^A and σ^A-RPases on promoter DNA melting indicated that core RPase, by sensing the presence of Mg^{2+}, is able to assist in the melting of the promoter −10 element.

DISCUSSION

We have provided direct evidence that SND100-σ^A by itself is able to bind promoter DNA in both specific and

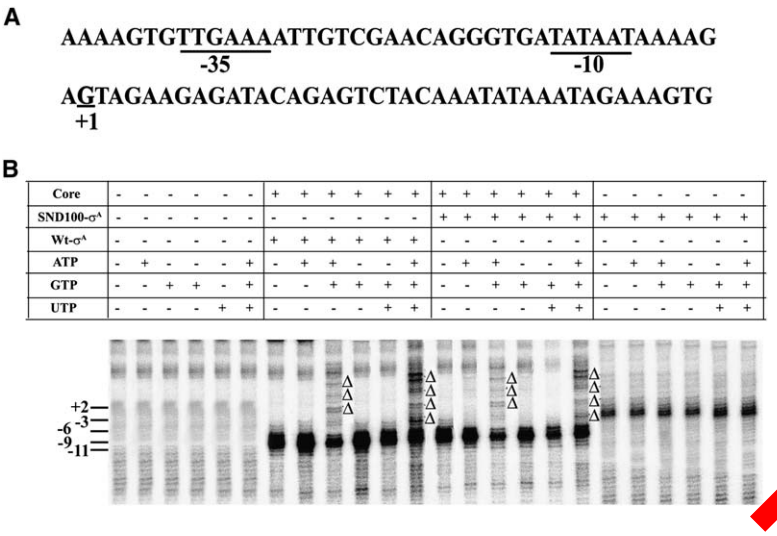

Figure 6. Effect of Nucleotide on Propagation of Promoter Melting by σ^A-RPases

(A) The DNA sequence of G3b promoter. The sequences of the promoter −10 and −35 elements and transcription start site (as indicated by +1) are underlined. The transcription start site, predominantly G, was determined by primer-extension assay using in vitro transcribed mRNA as the template (see Figure S3).

(B) Effect of NTP on propagation of promoter melting. The concentration of NTP was 0.25 mM when used. The molar ratio of σ^A-RPases to SND100-σ^A to G3b promoter DNA in the binding mixture was 400:1:1 or 800:1:1, respectively. The numbers shown on the left are positions relative to the transcription start site of the promoter.

nonspecific manners (Figure 1) and has a specific footprint on the promoter DNA (Figure 2). The specific σ^A binding requires the same promoter elements as those needed by σ^A in the context of RPase (Figure 3). Moreover, the specific σ^A binding to promoter DNA is enhanced by core RPase (Table S1). The ability of SND100-σ^A, lacking in the highly negatively charged region 1.1, to bind promoter DNA combined with the findings of segmental isotopic labeling (Camarero et al., 2002) and distance-constrained docking studies (Mekler et al., 2002) strongly support a view that region 1.1 masks the promoter-specific interaction of σ electrostatically and that the association of σ and core RPase essential this inhibition by relocating region 1.1 away from a location near the DNA binding surfaces of σ and into the positively charged DNA binding cleft of RPase. Thus, core RPase may direct promoter-specific binding of σ simply by changing the spatial positions of region 1.1 and the two DNA binding domains of σ so that the inhibitory effect of region 1.1 is neutralized without allosteric changes to the other σ regions. The capability of SND100-σ^A to bind DNA nonspecifically (K_d = ~2 × 10^{-6}) (Table S1) indicates that the mutant σ^A can be trapped by the noncognate sites that are ubiquitous on the chromosome, which has the effect of lowering the efficiency of specific promoter recognition and thus transcription efficiency. Our inability to develop a strain of *B. subtilis* using only this kind of *sigA* gene is consistent with this idea (Hsu et al., 2004). This phenomenon may also explain why multiple switches have to be introduced into the intact σ^A in order to control its DNA binding function.

It has been reported that the specific σ-initiated melting of promoter DNA requires core RPase or the coiled-coil σ binding motif of β′ subunit of the core RPase, which induces an allosteric change in σ (Young et al., 2004). In contrast to the above view, the mutant σ^A is capable of initiating direct promoter DNA binding and melting independent of core RPase once the inhibitory domain, region 1.1, is removed (Figures 2 and 4). This indicates that the allosteric change in σ as induced by core RPase or the coiled-coil motif of β′ is unnecessary for SND100-σ^A to function. Thus, SND100-σ^A, by itself, is a "melting protein" that is able to destabilize the double-stranded DNA in a thermodynamic manner (Jensen and von Hippel, 1976). However, melting of the promoter DNA by σ^A appears to be mechanistically related to that mediated by σ in the context of RPase since at least three identical aromatic amino acid residues are required by σ^A and σ^A-RPase to melt the G3b promoter DNA (Figure 5). Interestingly, the region melted by σ^A, which encompasses the transcription start site, is not available in the binary complex formed by G3b promoter DNA and σ^A-RPase (Figure 4B). Core RPase is thus able to negatively modulate the accessibility of the transcription start site at least on the G3b promoter DNA. To make the transcription start site accessible, subsequent nucleotide-dependent propagation of promoter melting is required (Figure 6B). In other words, and consistent with the proposal of other research (Brodolin et al., 2005), the σ^A-RPase melts promoter DNA in two separate steps: the initial melting of the promoter −10 element and the subsequent opening of the transcription start site during transcription initiation. However, the specific mechanism responsible for the requirement of both the −1 and +1 nucleotides for propagation of G3b promoter melting to the transcription start site remains obscure.

The temperature independence of SND100-σ^A melting of promoter DNA (Figure 7A) indicates that only the entropic driving force (or redistribution of water and salt ions) (Record et al., 1976; Spolar and Record, 1994) is accompanied with the specific σ^A-promoter DNA binding and melting. This is different from the temperature-dependent promoter melting accomplished by SND100-σ^A-RPase or WT-σ^A-RPase (Figure 7A), in which both entropy and enthalpy changes are involved (Roe et al., 1985; Straney and Crothers, 1985). The change in enthalpy may result from reduced exposure of the nonpolar surface upon interaction between core RPase and promoter DNA

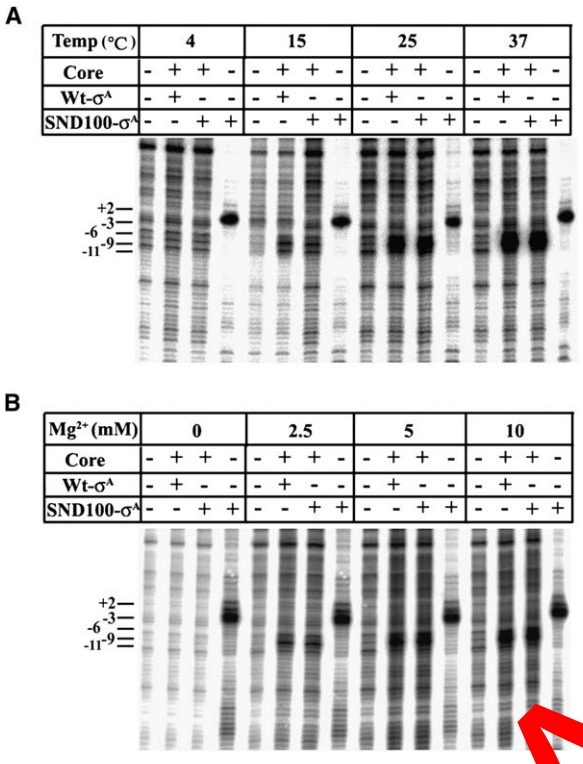

Figure 7. Effects of Temperature and Mg^{2+} on Base Modification of G3b Promoter DNA

(A) Effect of temperature on efficiency of promoter melting. Open complex II formed specifically in the presence of 0.6 μM heparin was incubated at a designated temperature (4°C, 15°C, 25°C, 37°C) and then probed with $KMnO_4$ at the same temperature.

(B) Effect of Mg^{2+} on efficiency of promoter melting. The concentration of Mg^{2+} tested was 0, 2.5, 5, and 10 mM. The binding reaction and $KMnO_4$ probing were performed at 37°C.

In both (A) and (B), the molar ratio of σ^A-RPase or SND100-σ^A to G3b promoter DNA was 10:1 or 800:1, respectively. The numbers shown on the left in both panels are the positions relative to the transcription start site of G3b promoter.

(hydrophobic effect). Thus, core RPase confers on σ^A-RPase the temperature-dependent promoter melting property. Similarly, the dependence on Mg^{2+} of promoter melting by σ^A-RPase indicates that core RPase is capable of sensing Mg^{2+} to adjust the melting of the promoter −10 element, probably through interaction between Mg^{2+} and the carboxylate triad of core RPase, which is essential for phosphodiester bond formation (Craig et al., 1995). All these results demonstrate that, in addition to the DNA binding and catalytic functions, core RPase also plays an important role in modulating the σ-initiated promoter binding and melting.

We believe that our finding of the novel *B. subtilis* mutant σ^A will prove useful for further examination of the detail functional mechanism of σ in recognition and melting of promoter DNA, and for further clarifying the role of core RPase during transcription initiation.

EXPERIMENTAL PROCEDURES

Overproduction and Purification of σ^A

Methods used for overproduction and purification of the N-terminally truncated σ^A were similar to those used for the preparation of WT-σ^A (Chang and Doi, 1990). The cell lysate was centrifuged at 7500 xg to harvest the σ^A aggregated in the inclusion bodies. The inclusion σ^A were then denatured with TEDG buffer (10 mM Tris-HCl, 1 mM EDTA, 1 mM DTT, and 5% glycerol) containing 6 M guanidine-HCl and refolded through stepwise dilution (10-fold) with TEDG buffer supplemented with 200 mM NaCl. The refolded σ^A was centrifuged at 7500 × g to remove the unfolded protein. The soluble σ^A in the supernatant was precipitated by 40% (w/v) ammonia sulfate and resuspended in TEDG buffer containing 200 mM NaCl before further purification with the gel filtration columns, Superdex HR 200 and Superdex SD-75.

Mutagenesis of *sigA* and G3b Promoter DNA

Mutant *sigA* with substitution of F178A, Y180A, Y184A, F186A, Y189A, W192A, or W193A was constructed using the QuickChange Site-Directed Mutagenesis Kit purchased from Stratagene. The plasmid, pSND100, having the coding sequence of SND100-σ^A, was used as a DNA template for mutagenesis. The primers used for amino acid substitutions are as follows: F178A-F: 5′-gcctgatgaaagccgttgaaaaagct attatcgcaaagg-3′ and F178A-R: 5′-cctttgcgataatcagcttttcaacggctttatcaggc-3′. Y180A-F: 5′-gaaagccgttgaaaaatttgatgctcgcaaaggttatattcagtacg-3′ and Y180A-R: 5′-cgtactgaatttataacctttgcgagcatcaaacaacggctttc-3′. Y184A-F: 5′-gccgttgaaaaatttgattatcgcaaaggtgctaaatcagg-3′ and Y184A-R: 5′-catacgtactgaatttagcacctttgcgataatcaaatttttcaacggc-3′. F186A-F: 5′-cgcaaaggttataaagccagtacgtacgtggtgg-3′ and F186A-R: 5′-ccaccacgtagcatacgtactggcttttaacctttgcg-3′. Y189A-F: 5′-ggttataaattcagtacggctgctacgtggtggatcagac-3′ and Y189A-R: 5′-gtctgatccaccacgtagcagccgtactgaatttataacc-3′. W192A-F: 5′-cagtacgtatgctacggcgtggatcagacaggc-3′ and W192A-R: 5′-gcctgtctgatccacgccgtagcatacgtactg-3′. W193A-F: 5′-gtacgtatgctacgtgggcgatcagacaggcg-3′ and W193A-R: 5′-cgcctgtctgatcgcccacgtagcatacgtac-3′. All of the *sigA* mutants were sequenced to be correct.

Mutations in the −10 element, TG motif, and −35 element of G3b promoter (Figure 3A) were also created using the same QuichChange Mutagenesis system. The plasmid, pCT-G3b, which contained the WT G3b promoter, was used as a DNA template for constructing the −10, TG, and −35 mutations. The primers used were as follows: −10 mutation, −10F: 5′-gaaaagtgttgaaaattgtcgaacagggtgaggtaacaaaagagtagaagag-3′ and −10R: 5′-ctcttctactcttttgttacctcaccctgttcgacaattttcaacactttc-3′. TG mutation, TG1F: 5′-gaaaagtgttgaaaattgtcgaacagggaatatataataaaagagtagaagag-3′ and TG1R: 5′-ctcttctactcttttattatattcccctgttcgacaattttcaacactttc-3′. −35 mutation, −35F: 5′-gtcgaacttttatagaaaagtgggaaaaattgtcgaacagggtgatataataaaagagtagaagag-3′ and −35R: 5′-ctcttctactcttttattatatcaccctgttcgacaattttcccacttttctataaaaagttcgac-3′. The pCT-G3b-derived plasmid, which harbors mutation at the −10 element, was used as a DNA template for −10/TG mutation. The two primers were TG2F: 5′-gaaaagtgttgaaaattgtcgaacaggggaaggtaacaaaagagtagaagag-3′ and TG2R: 5′-ctcttctactcttttgttaccttcccctgttcgacaattttcaacactttc-3′. All of the promoter mutants were sequenced to be correct.

In Vitro Transcription Assay

For in vitro transcription, 10 μl of core RPase (1 μg) prepared according to a previously reported protocol (Hsu et al., 2004) was mixed with an equal volume of the purified σ^A (1.2 μg) and incubated on ice for 10 min. The molar ratio of core to σ^A for RPase reconstitution was 1:10. Afterwards, 20 μl (0.3 μg) of pCT24 plasmid (Liao et al., 1999) harboring the G3b promoter of *B. subtilis* ϕ29 phage was mixed with the reconstituted RPase, and 40 μl of reaction cocktail (40 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 160 mM KCl, 0.4 mM DTT, 0.2 mM each of UTP, CTP, GTP, and ATP, 3 μCi [α-^{32}P] ATP, and 5% of glycerol),

prewarmed at 37°C, was added to start the transcription reaction. The reaction was allowed to proceed for 10 min before adding 160 μl of stop buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol). The mRNA products were run on a denaturing polyacrylamide gel and autoradiography was performed after the electrophoresis.

Electrophoretic Mobility Shift Assay

For the electrophoretic mobility shift assay (EMSA), the σ^A or σ^A-RPase was incubated with 1 nM ^{32}P-labeled G3b promoter DNA in 15 μl of DNA binding buffer (30 mM Tris-HCl, pH 7.9, 1 mM β-mercaptoethanol, 0.5 mM EDTA, 160 mM KCl, 10 mM $MgCl_2$, and 5% (v/v) glycerol) in the presence of 0.1 μM heparin (or as indicated) for 5 min at 37°C and then run on a native polyacrylamide gel in 1× TAE buffer (40 mM Tris-acetate, pH 8.5, and 2 mM EDTA) at 4°C. The heparin (Sigma H3393) used has a molecular weight of about 6 × 10^3 g. Finally, the gel was dried and analyzed with a Fuji BAS 2500-phosphoimager.

DNase I Footprinting Assay

The σ^A or σ^A-RPase was incubated with 40 nM ^{32}P-labeled G3b promoter DNA in 40 μl of DNA binding buffer for 5 min at 37°C. Then, 0.01 unit of DNase I (Roche) was added to digest the DNA. One minute later, 40 μl of stop buffer (50 mM Tris-HCl, 20 mM EDTA, 0.6 M NaOAc, and 10 μg of glycogen) was added to stop the digestion reaction. The digested DNA was extracted with phenol-chloroform. The DNA fragments in the upper aqueous layer were collected and precipitated with 0.3 M NaOAc, washed twice with 70% ethanol, heat dried (50°C), dissolved in 10 μl of loading buffer (98% deionized formamide), and electrophoresed with an 8% (w/v) sequencing gel in 1× TBE buffer (90 mM Tris-Boric acid, pH 8.5, and 2 mM EDTA).

$KMnO_4$-Primer Extension Analysis

The σ^A or σ^A-RPase was incubated with 50 ng of G3b promoter DNA in 17.5 μl of binding buffer (50 mM Tris-HCl, pH 7.9, 1 mM β-mercaptoethanol, 0.1 mM EDTA, 160 mM KCl, 10 mM $MgCl_2$, and 5% (v/v) glycerol) in the presence of 0.6 μM heparin for 5 min at the designed temperature. Then, 2.5 μl of $KMnO_4$ (final 1 mM) was added to treat the DNA at the same temperature for 2 min before adding 1 μl of 13.3 M β-mercaptoethanol to stop the reaction. The $KMnO_4$-treated DNA was purified through phenol-chloroform extraction and Sephadex G-25 spin column. For primer extension, oligonucleotides (BC1041-2: 5′-GAGCTCGGATCCAGAACG-3′ and BC1042: 5′-CTGCAGAAGCTTGCCATTTC-3′) complementary to the template and nontemplate DNA, respectively, were labeled with [γ-^{32}P]-ATP at their 5′ ends. Each of the reaction mixture (50 μl) contains the purified $KMnO_4$-treated DNA, primer (10^4 cpm), 0.5 mM dNTPs, and 0.25 units Taq DNA polymerase. After 25 extension cycles (1 min at 94°C, 1 min at 50°C, and 0.5 min at 72°C for each cycle), 10 μl of stop buffer (50 mM Tris-HCl, 20 mM EDTA, 0.6 M NaOAc, and 10 μg of glycogen) was added into the reaction mixture. The DNA was precipitated with ethanol, washed once with 70% ethanol, dissolved in 15 μl of loading buffer (98% deionized formamide), and electrophoresed with an 8% (w/v) sequencing gel in 1× TBE buffer.

Supplemental Data

Supplemental Data include six figures and one table and can be found with this article online at http://www.cell.com/cgi/content/full/127/2/317/DC1/.

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